VESSEL AND CULTURE SYSTEM INCLUDING THE VESSEL

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application is a U.S. National Phase Patent Application that claims the benefit under 35 U.S.C. § 365 of International Application No. PCT/GB2004/003352, filed August 3, 2004, which claims priority to Great Britain Application No. 0318411.6, filed August 6, 2003, the entire contents of each of which are hereby incorporated by reference herein.

BACKGROUND

Field of the Invention

[0002] The present invention generally relates to culture vessels. More particularly, the present invention relates to a vessel that includes a gas reservoir and at least one gas outlet, in which the gas outlet includes an integral gas permeable membrane and a culture system including the vessel.

Background Information

[0003] Micro-propagation encompasses a range of tissue culture methods for the propagation of plant species. In essence, tissue from a plant (explant) is isolated to create a sterile culture of that species *in vitro*. Once a culture is stabilized and growing well, multiplication of the tissue or regeneration of entire plants can be carried out. Shoots (tips, nodes or internodes) and leaf pieces are commonly used, but cultures can be generated from many different tissues. Such a method of cultivation of plant material is generally used for

rapid, large-scale, year round production of desired horticultural varieties; propagation of plant species that are difficult to grow from seed; production of genetically uniform plant material ("clones:); development of plant culture systems that can be used for genetic transformation, e.g., to introduce disease resistance and production of disease-free plant material, and other like uses.

[0004] Culture vessels currently employed for plant micro-propagation allow only poor ventilation and inadequate supplies of carbon dioxide to the cultures, so that at best the plants only photosynthesize at very low rates, as described in, for example, the following: Argita et al., "Influence of CO_2 and Sucrose on Photosynthesis and Transpiration of *Actinidia deliciosa* Explants Cultured *in vitro*," *Physiologia Plantarum* 115, pp. 166-173 (2002); Buddendorf-Joosten et al., "Components of the Gaseous Environment and Their Effects on Plant Growth and Development *in vitro*," *Plant Growth Regulation* 15, pp. 1-16 (1994) (hereinafter "Buddendork-Joosten, 1994"); Kozai et al., "Photoautotrophic and Photomixotrophic Growth of Strawberry Plantlets *in vitro* and Changes in Nutrient Composition of the Medium," *Plant Cell Tissue & Organ Culture* 25, pp. 107-115 (1991) (hereinafter "Kozai, 1991"); Kozai et al. in *Collected Papers on Environmental Control in Micropropagation*, Vol. 3 (Genhua Niu, ed.), Laboratory of Environmental Control Engineering, Faculty of Horticulture, Chiba University, Chiba 271, Matsudo, Japan (1995) (hereinafter "Kozai, 1995"); and Kozai et al., "Net Photosynthetic Rates of Plantlets *in vitro* Under Natural and Forced Ventilation Conditions," Annual Meeting, Japanese Society of Horticultural Science, pp. 250-251 (1989). Poor ventilation can lead also to accumulations of the gaseous hormone ethylene which may cause vitrification and other abnormalities in sensitive species, as described in, for example,

E.F. George, "Plant Propagation by Tissue Culture," Exogenetics Ltd., Edington, England (1995) (hereinafter "George, 1995").

[0005] While some photosynthesis may be supported by CO_2 diffusion under the lid of the culture vessel from the growth room atmosphere, unless the plants are maintained under continuous illumination, net daily photosynthesis may not be possible. This necessitates the addition of sugar to the nutrient medium that sometimes induces plant abnormalities and encourages the growth of contaminants, leading to considerable plant losses and increased costs of producing plants, as described in, for example, George, 1995. Furthermore, plants deprived of adequate levels of CO_2 may develop characteristics that lead to heavy losses at weaning to the glasshouse. These symptoms include: (a) dysfunctional, gaping, stomata that tend to cause excessive water loss from the plant; (b) insufficient food reserves (e.g., starch); and (c) poor lignification (woodiness). One of the major aims of the micro-propagation industry worldwide is to promote the growth of fully photosynthesizing plants in the laboratory, and so reduce or eliminate the need for additions of sugar to the nutrient medium, as described in, for example, the following: Figueira et al., "Optimizing Carbon Dioxide and Light Levels During *in vitro* Culture of *Theobroma cacao*," J. Amer. Soc. Hort. Sci. 119, pp. 865-871 (1994); George, 1995; Kozai, 1991; and Kozai, 1995.

[0006] The invention disclosed in Great Britain Patent No. 2,275,052 attempted to overcome the problem of poor ventilation in micro-propagation vessels by providing a ventilation apparatus and system for ventilating plant tissue cultures. The apparatus includes a chamber having a wall made from microporous membrane and a means for maintaining a water vapour partial pressure inside the chamber exceeding that outside the chamber. This

partial pressure differential induces a diffusive flow of atmospheric gases across the microporous membrane generating a positive pressure inside the chamber. An outlet discharges a continuous flow of humidified air from the chamber into a culture vessel. However, while the system reduces ethylene accumulation, the rate of flow is often insufficient to maintain a high enough concentration of CO_2 in the culture vessels to keep pace with the scavenging demands of the plants. The situation can be partly remedied by increasing the flow potential of the invention disclosed in Great Britain Patent No. 2,275,052, but unrealistically high rates of flow may be needed even to raise culture vessel CO_2 concentrations to atmospheric levels. An alternative and more practical method is to enrich the ventilating stream itself with CO_2.

[0007] Conventional methods of delivering CO_2 to cultures in plant micro-propagation rely on complex systems involving gas cylinders, pumps, regulators, gas mixers and filters, as described in, for example, Buddendorf-Joosten, 1994. Therefore, a need exists for a simple, inexpensive, portable method of delivering a sterile enriched gas to a culture vessel.

SUMMARY OF THE INVENTION

[0008] According to a first aspect of the present invention, there is provided a vessel including a gas reservoir and at least one gas outlet, in which the gas outlet includes an integral gas permeable membrane.

[0009] Preferably, the gas diffuses across the gas permeable membrane. This differs from the conventional gas cylinders, for example, CO_2, in which the gas is transported by

convection and a needle valve is required to control the bulk flow rate from the cylinder. The rate of diffusion across the gas permeable membrane is dependent on the membrane's diffusive resistance which can be varied by altering the physical properties of the membrane. Such alterations can be achieved by altering the surface area or nature of the membrane and/or by using a membrane of different wall thickness. The membrane can consist of silicon rubber (Si-rubber), that has a CO_2 permeability coefficient, P_{si}, of ca. 2.28×10^5 cm^2 s^{-1} at 20°C or other appropriate materials with a suitable diffusive resistance. The effective diffusive resistance, R_D, of an annulus of membrane length L is given by Equation (1):

$$R_D = (r_i \log_e r_o/r_i)/P_{si}2\pi iL \qquad (1)$$

where r_i and r_o are the inner radii of the Si-rubber tube. Similarly, the effective diffusive resistance for other gases can be determined by using their respective permeability coefficients in Equation (1).

[0010] In a preferred embodiment of the present invention, the gas can be a fluid. Even more preferably, the fluid can be a gas that is selected from the group consisting of, for example, O_2 or CO_2. In an alternative exemplary embodiment of the invention, the fluid can be a liquid. Even more preferably, the gas reservoir can be enriched for at least one gaseous species. Preferably still, the gas reservoir can be liquid enriched for CO_2, for example, carbonated water or a solution of buffered bicarbonate salt that enriches for CO_2. During culture, the vessel can be linked to at least one substantially sealed culture vessel. The concentration of CO_2 in the gas reservoir diminishes during the culture period, as a result of use by the culture and leakage from the culture vessel. Such diminishment is particularly advantageous during the micro-propagation of plants as it essentially "weans" the plants off

high concentrations of CO_2 so that at the completion of the micro-propagation phase they are able to readily adapt to atmospheric CO_2 levels.

[0011] If, however, the rate of diffusion of CO_2 across the gas permeable membrane is to be maintained or increased during culture, this may be achieved by: (i) the use of more than one gas permeable membrane per vessel; (ii) the replacement of a membrane with a high diffusive resistance to one with a lower diffusive resistance; or (iii) the use of a membrane with the potential to alter its diffusive resistance in response to conditions in the culture vessel.

[0012] In a further exemplary embodiment of the present invention, the gas is sterilized as it diffuses across the gas permeable membrane.

[0013] In a still further exemplary embodiment of the invention, the gas reservoir includes more than one gaseous species. For example, the gas reservoir can include CO_2 and a gaseous ethylene inhibitor. Ethylene is a very potent gaseous hormone produced by plants, and can at times be toxic in plant micro-propagation cultures. It is frequently cited as a cause of abnormal growth or vitrification, including stunting, leaf curling and premature shoot senescence in sensitive species, as described in, for example, the following: George, 1995; and Righetti et al., "Ethylene, Ethanol, Acetaldehyde and Carbon Dioxide Released by *Prunus avium* Shoot Cultures," *Physiologia Plantarum* 78, pp. 507-510 (1990). The gaseous ethylene inhibitor 1-methyl cyclopropene (1-MCP), available from, for example, Rohm & Haas Co. (Pennsylvania, USA), is an extremely specific inhibitor of ethylene action and works by binding specifically to the sites of ethylene action within the plant. By providing a

1-MCP solution in the gas reservoir, the present invention can be used to enrich the culture vessel with gaseous 1-MCP, and thus prevent vitrification.

[0014] According to a further aspect of the present invention, there is provided a culture system including a vessel according to the present invention, in which the vessel is connected to a second vessel comprising a cell. Preferably, this is a plant cell. Even more preferably, the plant is undergoing micro-propagation. Alternatively, the cell is selected from, for example, an animal, bacterial or yeast cell.

[0015] In a further aspect of the invention, there is provided a culture system including a first vessel according to the present invention that is connected to a second vessel that contains a plant.

[0016] The vessel of the present invention can also be utilized for medical applications, specifically veterinary applications, in which it can be used as part of a ventilation device/system to deliver and/or enrich oxygen to small animals/insects located within a second vessel. Thus, according to a still further aspect of the invention, there is provided a ventilation system including a first vessel according to the present invention that is connected to a second vessel that contains an animal.

[0017] The vessel according to the present invention can be connected by way of interconnecting means to more than one culture vessel.

[0018] Following diffusion through the gas permeable membrane, the gas is transported towards the culture vessel by diffusion. This is suitable for plants that are not ethylene sensitive, and, therefore do not require a ventilation stream through the culture vessel. Alternatively, for plants that are ethylene sensitive and where a gaseous ethylene inhibitor is not being used, flushing out of potentially toxic gaseous products from the culture vessel can be achieved by further connecting a means of convective, pressurized delivery to the culture system. Thus, in a further exemplary embodiment of the invention, the culture system is further adapted to connect with a pressurized ventilation stream. For example, an interconnection between the first and second vessel, preferably in the form of a pipeline, can be adapted to connect with the pressurized ventilation stream. The connection can be to the outflow tube of a pressure-flow source, such as a humidity-induced forced ventilation apparatus described in, for example, Great Britain Patent No. 2,275,052, or any other suitable filtered air source.

[0019] In a culture system reliant on the simple diffusive delivery of the gas, the rate of flow of the gas from the gas reservoir to the culture vessel is dependent on the factors, including, for example, (i) the diffusive resistance of the gas-permeable membrane, and (ii) the diffusive resistance of the connection, as well as the diffusive resistance under the rim.

[0020] In a culture system reliant on convective, pressurized delivery, the rate of flow from the gas reservoir to the culture vessel depends chiefly on, for example, (i) the diffusive resistance of the gas-permeable membrane, and (ii) the rate of convective gas flow from the pressurized ventilating source.

[0021] According to a further aspect of the present invention, a method is provided for the supply of a gaseous species to a cell that includes the steps of: (i) providing a vessel comprising a gas reservoir and at least one gas outlet wherein the gas outlet comprises a gas-permeable membrane; (ii) connecting, via an interconnecting means, the vessel to at least a second vessel comprising a cell; and, optionally, (iii) further connecting a humidity-induced forced ventilation apparatus to the interconnecting means. Preferably, the method is used in the supply of a gaseous species to a cell. Preferably, this cell is a plant cell. Even more preferably, this plant cell is undergoing micro-propagation. In an alternative preferred method, a gaseous species is supplied to a cell selected from the group consisting of, for example, an animal, bacterial or yeast cell.

BRIEF DESCRIPTION OF THE DRAWINGS

[0022] Other objects and advantages of the present invention will become apparent to those skilled in the art upon reading the following detailed description of preferred embodiments, in conjunction with the accompanying drawings, wherein like reference numerals have been used to designate like elements, and wherein:

[0023] FIGS. 1A and 1B are schematics of the culture apparatus for delivery of gas from a vessel including a gas reservoir to a culture vessel by simple diffusive flow (Method A), in accordance with an exemplary embodiment of the present invention.

[0024] FIGS. 2A and 2B are schematics of the culture apparatus for delivery of gas from a vessel comprising a gas reservoir to a culture vessel by convective, pressurized flow (Method B), in accordance with an exemplary embodiment of the present invention.

[0025] FIG. 3 illustrates examples of gas delivery by convective, pressurized flow [Method (B)] when there is not a sample in the culture vessel, in accordance with an exemplary embodiment of the present invention.

[0026] FIG. 4 is graphs that are derived from mathematical modeling that illustrate how the diffusive resistance of the gas permeable membrane (as a function of its length) affects photosynthesis of micro-propagated Cherry when applying CO_2-enrichment by Method (A) and Method (B), in accordance with an exemplary embodiment of the present invention.

[0027] FIG. 5 is graphs that illustrate details of CO_2 supply rate and escape rate of unused CO_2 from the culture vessels outlined in FIG. 4, in accordance with an exemplary embodiment of the present invention.

[0028] FIG. 6 is a graph that illustrates daily net photosynthesis of Cherry grown on multiplication media either with (a) forced ventilation in conjunction with CO_2 enrichment from the culture apparatus of the present invention, (b) forced ventilation without CO_2 enrichment or (c) conventional diffusive ventilation, in accordance with an exemplary embodiment of the present invention.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0029] FIG. 1A is a schematic that illustrates the culture system 1 when used for simple, diffusive delivery of a gas. The vessel 2 comprises a gas reservoir 3 sealed from the atmosphere by the vessel 2 having a gas-tight, screw-top lid 4. This gas reservoir 3 can be, for example, carbonated water, a solution of buffered bicarbonate salt (e.g., sodium bicarbonate) or the like. A gas outlet 5 with an integral gas permeable membrane 6 is positioned in the head space of the vessel 2. The lower end of the gas outlet 5 is sealed with a blanking plug of glass or other suitable material 7. As the gas diffuses across the membrane 6 and out of the vessel 2, it enters an interconnecting means 8 and diffuses in the direction of the arrows towards a culture vessel 9.

[0030] FIG. 1B is a schematic that illustrates a magnified view of the gas outlet 5 extending from the vessel 2 comprising the gas reservoir 3. In this exemplary embodiment, the gas outlet 5 can be comprised of, for example, a T-piece with a vertical limb 5a and a horizontal limb 5b. When in use, the end of horizontal limb 5b that is distal to the culture vessel 9 can be sealed by means of a blanking screw 10 or the like, while the end that is promixal is attached by a screw connector 11a or the like to the interconnecting means 8 (e.g., a plastic tube, pipe or the like), of low wall permeability to gases, that extends through the lid of the culture vessel 9.

[0031] FIG. 2A is a schematic that illustrates the culture system 1 when using convective, pressurized gas delivery. As the gas diffuses through the gas outlet 5, it mixes with the outflow from a pressure-flow source 12, such as a humidity-induced forced ventilation

apparatus or the like. The arrows indicate the direction of convective gas flow along the interconnecting means 8.

[0032] FIG. 2B is a schematic that illustrates a further exemplary embodiment of the present invention and shows a magnified view of the gas inlet 5 extending from the vessel 2 comprising the gas reservoir 3. In this exemplary embodiment, the gas outlet 5 can be comprised of, for example, a T-piece with a vertical limb 5a and a horizontal limb 5b. When in use, the end of horizontal limb 5b that is distal to the culture vessel 9 is attached by means of a screw connector 11b or the like to the outflow of a pressure-flow source 12, such as a humidity-induced forced ventilation apparatus or other suitable apparatus. The end that is promixal is attached by a screw connector 11a or the like to the interconnecting means 8 (e.g., a plastic tube, pipe or the like), of low wall permeability to gases, that extends through the lid of the culture vessel 9.

[0033] FIG. 3 includes graphs that illustrate examples of gas delivery by convective, pressurized flow [Method (B)] when there is not a sample in the culture vessel. FIG. 3 illustrates the effects of increasing the rate of pressurized air flow through the T-piece on both CO_2 delivery rate and CO_2 concentration. A comparison is made with apparatus without a CO_2-enrichment device.

[0034] FIG. 4 illustrates graphs that are derived from mathematical modeling. FIG. 4 illustrates how the diffusive resistance of the gas permeable membrane (as a function of its length) affects photosynthesis of micro-propagated Cherry when applying CO_2-enrichment by Method (A) and Method (B). It can be seen that by both procedures, photosynthesis is

much enhanced above that obtained without CO_2-enrichment. With a gas permeable membrane length of, for example, 3 mm, photosynthesis is approximately 6.5 times greater than could be achieved by conventional treatments that rely on CO_2 diffusion from the growth room atmosphere under the lid of the culture vessel. However, with Method (a), more of the CO_2 used by the plants is derived from the reservoir than with Method (b), where the pressurized gas flow itself can contain some CO_2. In the examples shown, the pressurized gas stream contains atmospheric levels (e.g., 360 ppm). As the gas permeable membrane length is increased (which lowers its diffusive resistance), Method (b) removes increasingly more CO_2 from the reservoir than Method (a) and proportionally less of it is used in photosynthesis. Overall, Method (b) can be more wasteful of CO_2 than Method (a), but this is unavoidable if a pressure flow gas stream is also necessary to remove undesirable volatiles from the culture vessels.

[0035] FIG. 5 illustrates details of CO_2 supply rate and escape rate of unused CO_2 from the culture vessels outlined in FIG. 4. Overall, Method (b) can be more wasteful of CO_2 than Method (a), but this is unavoidable if a pressure flow gas stream is also necessary to remove undesirable volatiles from the culture vessels.

[0036] FIG. 6 illustrates daily net photosynthesis of Cherry grown on multiplication media either with (a) forced ventilation in conjunction with CO_2 enrichment from the culture apparatus of the present invention, (b) forced ventilation without CO_2 enrichment, or (c) conventional diffusive ventilation. When used for CO_2 enrichment, the culture system according to exemplary embodiments of the present invention stimulates photosynthesis.

[0037] Table 1 illustrates the beneficial effects obtained by applying the gaseous ethylene inhibitor, 1-methyl cyclopropene (1-MCP), to cherry using the culture apparatus of the present invention. In particular, Table 1 illustrates the effect of the ethylene inhibitor, 1-MCP, on vitrified curling of leaves, leaf senescence and abscission and shoot tip necrosis after 16 days.

[0038] It will be appreciated by those of ordinary skill in the art that the present invention can be embodied in various specific forms without departing from the spirit or essential characteristics thereof. The presently disclosed embodiments are considered in all respects to be illustrative and not restrictive. The scope of the invention is indicated by the appended claims, rather than the foregoing description, and all changes that come within the meaning and range of equivalence thereof are intended to be embraced.

[0039] All United States patents and applications, foreign patents and applications, and publications discussed above are hereby incorporated herein by reference in their entireties.